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STUDY OF MICROFLORA INVOLVED IN COCOA BEAN FERMENTATION IN TAMIL NADU

C. ASHOKKUMAR[1], D. BASKARAN[2] & K. S. GNANALAKSSHMI[3]

[1]*Research Scholar, College of Food and Dairy Technology, TANUVAS, Chennai, Tamil Nadu, India*

[2]*Dean, College of Food and Dairy Technology, TANUVAS, Chennai, Tamil Nadu, India*

[3]*Assistant Professor, College of Food and Dairy Technology, TANUVAS, Chennai, Tamil Nadu, India*

ABSTRACT

The microbial ecology of cocoa bean fermentation involves the succession growth of various species of yeasts, lactic acid bacteria (LAB), acetic acid bacteria (AAB) are mostly involved in traditional process of cocoa bean fermentation. High counts were detected at the beginning of the fermentation (5.7+ or - 0.26 cfu/g dry matter) and were present throughout fermentation time. The predominant species such as Yeast, Lactic Acid Bacteria and Acetic Acid Bacteria were isolated from the cocoa pulp using selective agar plates and the identification was carried out by both biochemical method and molecular methods. The yeast isolate shows pseudohyphal morphology on YPD agar plate. The colonies of Lactic acid bacteria (LAB) appeared as large, convex and glistening white colonies embedded in Lactobacilli MRS Agar plate. Acetic acid bacteria (AAB) appeared as smooth spheroid to a flattened aggregate with a characteristic pillowed surface surrounded by a sheath of cellulose microfibrils on D-mannitol agar plate. Naturally fermented cocoa fruit seed samples were analysed for the presence of Saccharomyces cerevisiae, Lactobacillus fermentum and Acetobacter aceti using species specific primers. Isolated DNA samples were PCR amplified with species specific primers and further confirmed with gel electrophoresis.

KEYWORDS: Cocoa, Yeast, LAB, AAB, Fermentation & Molecular Characterization

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Original Article

INTRODUCTION

Cocoa (*Theobroma cacao*) is native to Central and South America, but is grown around the world where environmental conditions are highly conducive. According to *The World Cocoa Foundation* the cocoa crop worldwide supports 40-50 million people who depend on it for their regular livelihood and 4-6 million farmers around the world grow it. Chocolate is a product, produced using the cocoa fruit seed, and obtained from the cocoa tree a family of malvaceae. Cocoa production in south India is about 15% which meets out 60 % of total demand for the product amongst consumers in our country. Cocoa cultivation in India especially in Tamil Nadu is gaining momentum during recent times though the concept of cocoa production has been seeded in the year 1950. Of late, the consumer's interest orienting towards inclusion of cocoa based products mainly chocolates in their food regimen owing to its health benefiting factors embracing the cocoa fruit. Cocoa fermentation was a key step in the technological transformation of cocoa into chocolate, because the highly bitter, astringent unfermented cocoa seeds lack the full chocolate flavour (Schwan *et al.*, 1995). Fermentation of cocoa seeds was the first step of the chocolate-making process, which consists of a natural, 5- to 7- day microbial fermentation of the pectinaceous pulp surrounding the seeds (Schwan *et al.*, 2004). Different microbes involved cocoa seed fermentation produce

various flavour characteristics which leads to control polyphenol concentration and quality of the seeds (Camu *et al.,* 2008). The presence of microorganisms during cocoa seed fermentation reflects the environmental changes (temperature, pH, and oxygen concentration) and the substrates concentration of ethanol, lactic acid, and acetic acid changes. After cocoa seed fermentation higher concentrations of glucose, fructose and minor concentrations of lactic and acetic acids present inside the seeds (Igor *et al*., 2013). Microorganism in cocoa fermentation contributes good chocolate quality and more chocolate flavor. Seeds fermented with yeast were fully brown in colour and gave chocolate with typical characteristic yeast growth and activity that was essential for cocoa seed fermentation.

The microbial ecology of cocoa seed fermentation is a complex process which involves the successive growth of various species of yeasts, lactic acid bacteria and acetic acid bacteria. Of the lactic acid bacteria, *Lactobacillus plantarum* and *Lactobacillus fermentum* are more frequently encountered, although contributions from Pediococcus and Leuconostoc species are sometimes reported. Under the acetic acid bacteria, *Acetobacter pasteurianus* is the most frequent main contributor, but other species are also involved including *Gluconobacter oxydans*, *Acetobacter tropicalis*, *Acetobacter lovaniensis* and *Acetobacter syzygii*. It is not fully understood how these microbial groups or individual species determine cocoa seed quality and chocolate character and, indeed, whether or not they are essential to the fermentation process. Identification of bacteria based on DNA, use of DNA sequencing, PCR, all of these methods rely on visualization of DNA bands they were from restriction digestion; hybridization or PCR amplification. These banding patterns or the 'DNA fingerprints' are used to compare one isolate with another. Most of the DNA fingerprinting techniques are based on the presence/absence of the restriction sites (polymorphic restriction sites) while others are based on the homologies to short oligonucleotide primers.

MATERIALS AND METHODS

Sampling and Fermentation

Cocoa fruit (*Theobroma cacao L*) Forastero variety (widely cultivated in India) was collected from cocoa farm located in Mathukoore village, Tanjore district, Tamil Nadu. Natural fermentation was carried out with 10 kg of freshly harvested cocoa fruit opened and placed in plastic basket covered with banana leaves and placed for 6 days natural fermentation.

MICROBIOLOGICAL ANALYSIS

Sample Preparation and Colony Enumeration

Sample was taken every 24 hours. Five gram of fermented cocoa seeds weighed and transferred into Erlenmeyer flask containing 100 ml of 0.1% peptone water and some sand for remove the mucilaginous pulp. Peptone water enriched sample was inoculated in YPD, MRS and D-mannitol agar plate for isolation and identification of Yeast, LAB and AAB respectively incubated at 25˚C, 30˚C and 37˚C for 24-48hrs. Well grown colony was further purified using selective antibiotic added agar plates incubated at appropriate temperature then purified colony was inoculated in antibiotic added broth and kept at appropriate temperature for 24 hr.

Morphological and Biochemical Confirmation

Gram staining can narrow down to identity cultures to gram-positive and gram negative classes, and then the cultures can be identified to a specific species by using the polymerase chain reaction (PCR). Catalase Test, Ethanol

Concentration Test, Temperature Tolerance Test, Flocculation Test, Hydrogen Sulfide Production Test, Sugar Fermentation Test were carried out according to (Ouattara *et al.*, 2008)

Molecular Identification

Polymerase Chain Reaction (PCR) was used for identifying bacterial species in fermented cocoa sample based on Dubernet *et al.*, (2002).

DNA isolated from fermented samples was screened using primers listed in the Table 1. The PCR reaction cocktail was prepared the total volume of 50µl. Master Mix 20 µl was dispensed into PCR tubes. Five µl of each diluted primer mix was added into the PCR tubes containing the Master Mix (i.e. 5 µl x 4 = 20 µl) plus 25 µl of distilled water was added into the PCR tubes. Finally 5 µl of template DNA (kept on ice) was added into the PCR tubes. The PCR tubes were then placed into the PCR (Eppendorf AG 22331 Hamburg) and run using the general procedure of (Poblet *et al.*, 2000).

PCR machine running profile for selected of *S. cerevisiae, L. fermentum and A. aceti* specific primer with different annealing temperature and cyclic condition was shown below.

Table 1

Primer Name	Sequence	Annealing	Amplicon	Reference
SC1	AACGGTGAGAGATTTCTGTGC	50˚C	1170bp	Josepa et al. (2000)
SC2	AGCTGGCAGTATTCCCACAG			
Lfpr	GCCGCCTAAGGTGGGACAGAT	60˚C	337bp	Walter *et al.* (2000)
FermII	CTGATCGTAGATCAGTCAAG			
F	TGGAGCATGTGGTTTAATTCGA	58˚C	88bp	Kantor., *et al* (2014)
R	GCGGGAAATATCCATCTCTGAA			

Sixty ml of 1X TAE (Tris Acetate and EDTA) buffer with 0.72g of agarose (1.2%) added was poured into a 100 ml flask and placed on a hot plate to boil. After boiling the solution became clear. After cooling 5 min the gel solution was poured into the gel tray to set. Two µl of nucleic acid dye (gel loading dye purple 3X dye (Bromophenol blue, xylene blue, formamide and NaOH)) and 8 µl of PCR sample added. Five µl of mix solution were withdrawn from the tube into the agarose gel and 5 µl of ladder (100 base pair standard) added into the gel. The gel was placed into the electrophoresis unit and 500 ml of 1X TAE buffer solution was poured into the tray. The electrophoresis conditions were 100 V for 1 hour and 45 minutes.

RESULTS AND DISCUSSIONS

Microbiological Analysis

Three predominant species of Yeast/LAB/AAB were isolated from the naturally fermented cocoa fruit seed using selective agar plate and gram staining technique. The results obtained were shown in Figure 1, 2 and 3.

The figure 1, 2 and 3 portraits the pink colour and spherical shape gram negative, violet colored rod shaped gram positive bacilli and pink colored spherical shape gram negative organisms respectively. The preliminary results confirmed that the fermentation of cocoa fruit seeds was initiated by yeast/LAB/AAB in its natural composition.

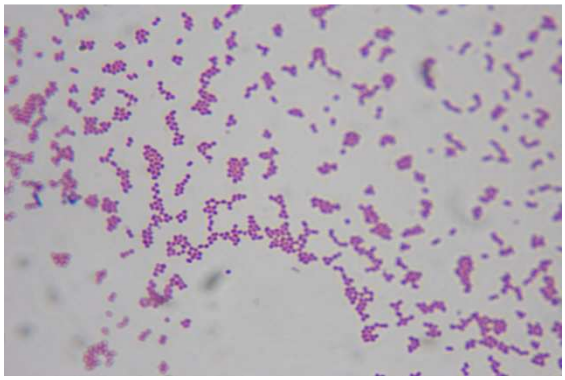

Figure 1: Microscopic Image of *Saccharomyces Cerevisiae*

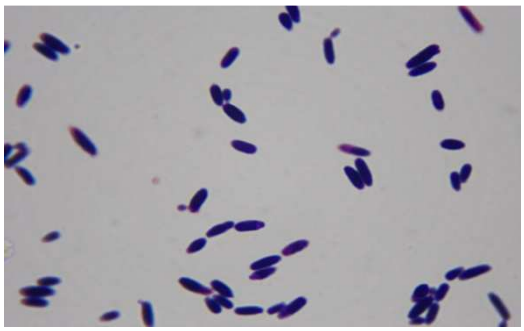

Figure 2: Microscopic Image of *Lactobacillus Fermentum*

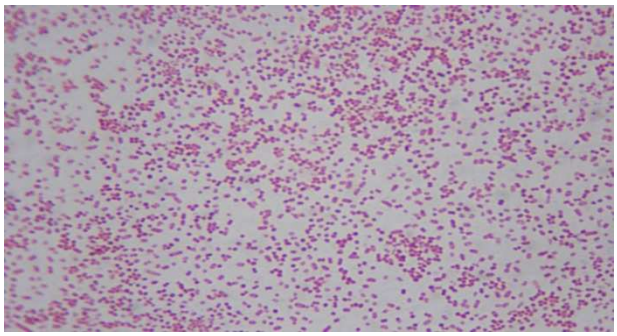

Figure 3: Microscopic Image of *Acetobacter Aceti*

The result showed that the rod shaped gram negative bacilli (Yeast), rod shaped gram positive bacilli (LAB) and spherical shaped gram negative organisms (AAB) were isolated using the selective agars following conventional method of plating. The identification of these organisms was later confirmed by its morphological features with straining technique. It was reported that these bacterial isolates were normally associated with the fermentation process and contributes for the characteristic flavor of the cocoa fruit seeds (Lefeber *et al.* 2012). The study conducted by De Melo Pereira *et al.* (2012) revealed the presence of yeast in the cocoa fruit seed and its pulp which were responsible for natural fermentation. According to Meersman *et al.* (2013), the normal composition of microbes in cocoa fruit pulp majorly encompasses lactic acid bacteria and acetic acid bacteria which are in conformity with the present investigation.

Biochemical Characterization

The yeast isolate shows pseudohyphal morphology on YPD agar plate. After determining that it was a gram negative rod, an ethanol concentration test, temperature tolerance test, flocculation test, hydrogen sulfide production test and sugar fermentation were performed and the results were listed in Table 2.

Table 2: Biochemical Confirmation of Yeast Colonies

S. No	Test	Result
1.	Gram stain	-
2.	Ethanol concentration test (g/L)	
	100	+
	130	+
	150	-
3.	Temperature tolerance test	
	25˚C	+
	37˚C	+
	45˚C	+
4.	Flocculation	-
5.	Hydrogen Sulfide	+
6.	Fermentation of sugars	
	Glucose	+
	Sucrose	+
	Maltose	+
	Galactose	+
	Raffinose	+
	Lactose	-

The colonies of Lactic acid bacteria (LAB) appeared as large, convex and glistening white colonies embedded in Lactobacilli MRS Agar plate. After determining that it was a gram positive rod, biochemical test such as catalase test, NH_3 from arginine, temperature tolerance test and sugar fermentation test were performed and the results were listed in Table 3.

Table 3: Biochemical Confirmation of LAB Colonies

S. No	Test	Result
1.	Gram stain	+
2.	Catalase	-
3.	NH3 from arginine	-
4.	Growth at temperature	
	10˚C	-
	15˚C	-
	37˚C	+
	45˚C	+
5.	Sugar fermentation test	
	Arabinose	-
	Fructose	+
	Galactose	+
	Glucose	+
	Lactose	+
	Mannose	-
	Sucrose	+
	Xylose	-
	Sorbitol	-
	Raffinose	-

Acetic acid bacteria (AAB) appeared as smooth spheroid to a flattened aggregate with a characteristic pillowed surface surrounded by a sheath of cellulose microfibrils on D-mannitol agar plate. After determining that it was a gram negative spherical, elongated, and club-shaped rod biochemical test such as carbon source and sugar fermentation tests were performed and the results were listed in Table 4.

Table 4: **Biochemical Confirmation of AAB Colonies**

S. No	Test	Result
1.	Gram stain	-
2.	Growth on carbon sources	
	Glycerol	+
	Ethanol	+
	Sodium acetate	+
3.	Fermentation of sugars	
	Glucose	+
	Mannose	+
	Galactose	+
	Xylose	+

In the present work, Yeast, LAB and AAB were isolated in the selective media and characterized by gram staining which were further ascertained by genus specific biochemical tests. Guimaraes *et al.,* (2006) reported that the yeast can withstand the ethanol tolerance limit of 130 g/L, which is obvious since yeast would produce ethanol during its growth phase while undergoing fermentation process. The temperature tolerance test showed that yeast can withstand the temperature of 45°C which was earlier identified by (Schwan, 1998). It was found that yeast can metabolize glucose, sucrose, maltose, galactose and raffinose as heterofermentative genera (Guimaraes *et al.,* 2006). In the case of LAB colonies, biochemical tests such as catalase, arginine, temperature tolerance and sugar fermentation tests were carried out. It was found that LAB were catalase negative and grown at 45°C, able to metabolize sugars viz. glucose, sucrose, galactose, fructose and lactose (Azadnia *et al.,* 2011). For the confirmation of AAB, certain biochemical tests viz. growth on glycerol, ethanol and sodium acetate, sugar fermentation tests such as glucose, mannose, galactose and xylose were carried out. The results showed that AAB could be able to ferment sugars such as glucose, mannose, galactose and xylose due to involvement of these organisms in the production of acetic acid with the ability to ferment carbon source as reported by Ukwo and Ezeama (2011).

Molecular Identification Isolates

Naturally fermented cocoa fruit seed samples were analysed for the presence of *Saccharomyces cerevisiae*, *Lactobacillus fermentum* and *Acetobacter aceti u*sing species specific primers. Isolated DNA samples were PCR amplified with species specific primers and further confirmed with gel electrophoresis. The gel electrophoresis image was given in the Figure 4. *Saccharomyces cerevisiae* were amplified during the first four days of fermentation at base pair size of 1170 bp lane 2-5 compared to the reference lane 7. *L. fermentum* isolates amplified from third day of fermentation at the base pair of 337 bp lane 4-6 compared with reference lane 7 showed in Figure 5. *A. aceti* isolates amplified from fourth day of fermentation at the base pair of 88 bp lane 5 and 6 compared with reference lane 7 showed in Figure 6.

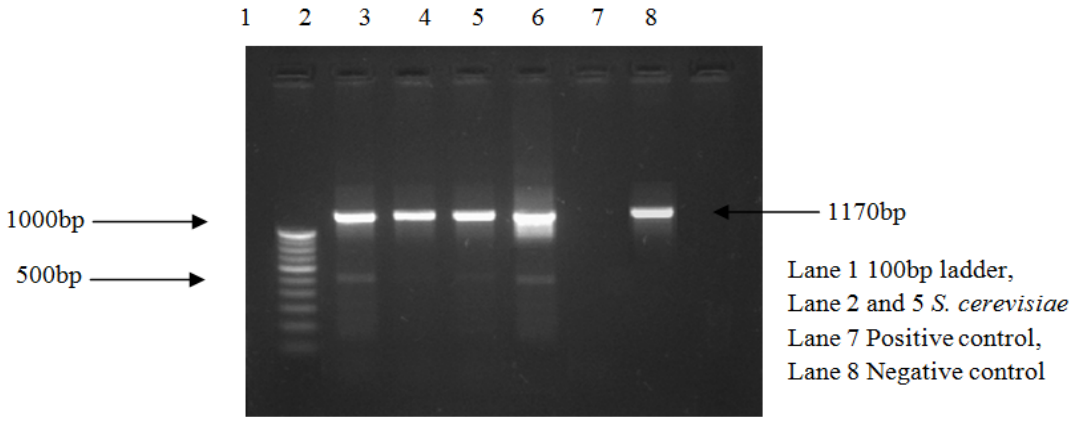

Figure 4: Electrophoresis on a 1.2% Agarose Gel of *S.cerevisiae* Isolate

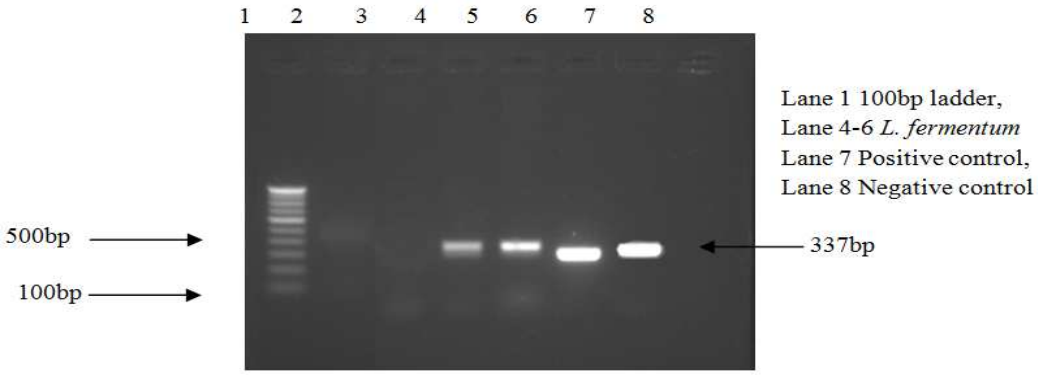

Figure 5: Electrophoresis on a 1.2% Agarose Gel of *L. fermentum* Isolate

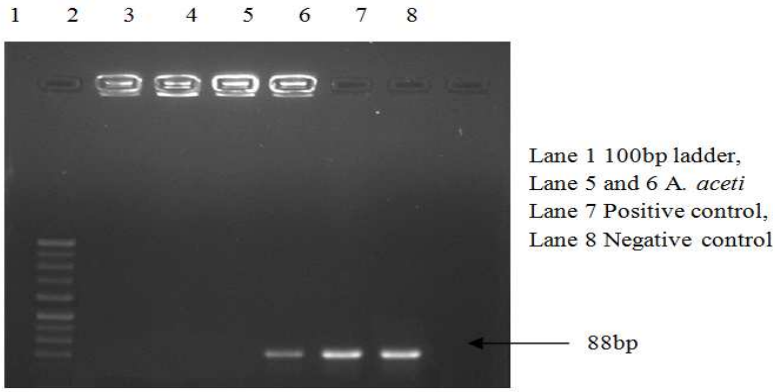

Figure 6: Electrophoresis on a 1.2% Agarose Gel of *A. aceti* Isolate

The organisms such as yeast, LAB and AAB were further identified using the molecular technique such as Polymerase chain reaction (PCR). Generally PCR involves the amplification of desired DNA molecule using the species specific primers which were selectively amplified and produced the corresponding base pair size products. The result obtained showed that the yeast was identified with 1170 base pair size products and this was in agreement with the study carried out by (Josepa *et al.*, 2000). The AAB which was amplified with the primer sequence and product size of 88 base pairs showed the positive result. For the confirmation of LAB, the primer sequence which can generate the product size of 337 base pairs was used that showed the positive result as evident from the gel electrophoresis. The molecular identification of yeast, AAB and LAB were reported in previous studies (Lefeber *et al.*, 2012) and the presence of these

three organisms in the present study confirmed their role in fermentation and development of characteristic flavor in cocoa fruit seed.

CONCLUSIONS

Cocoa beans fermentation is one of the essential step in flavour formation for the development of specific chocolate characters during further processing. The main microorganisms involved in cocoa fermentation are yeasts, lactic acid bacteria and acetic acid bacteria. A study was undertaken to identify various microbial isolates of yeast, LAB and AAB for the production of chocolates with a respective consistent chocolate flavour, independent of cocoa-producing region and fermentation method. The microbial groups selected for this study were *S. cerevisiae, L. fermentum* and *A. aceti* which were isolated from natural fermentation and utilized for formation of different culture combinations for cocoa fruit seed fermentation. The isolation of microbes and their identification were investigated by both conventional and molecular methods with the species specific primers amplified with DNA sample target region and electrophoresis on 1.2% polymerase chain reaction. Culture combinations were inoculated into cocoa fermentation processes - CC1 (*S. cerevisiae and L. fermentum*), CC 2 (*L. fermentum* and *A. aceti*), CC 3 (*A. aceti* and *S. cerevisiae*) and CC 4 (*S. cerevisiae, L. fermentum* and *A. aceti*).

REFERENCES

1. *Azadnia, P., M. H. Zamani, S. A. Ghasemi, A. K. Babaki, M. K. Jashni and N. Taarof. (2011). Isolation and Identification of Themophilic Lactobacilli from Traditional Yoghurts of Tribes of Kazerum. Journal of Animal and Veterinary Advances,* ***10(6)****: 774-776.*
2. *Camu, N., A. Gonzalez, T. De Winter, A. Van Schoor, K. De Bruyne, P. Vandamme, S. J Takrama, K. S. Addo and L. De Vuyst. (2008). Influence of turning and environmental contamination on the dynamics of populations of lactic acid and acetic acid bacteria involved in spontaneous cocoa bean heap fermentation in Ghana. Applied Environmental Microbiology,* ***74*** *(1): 86–98.*
3. *De Melo Pereira, G. V., M. G. da Cruz Pedrozo Miguel, C. L. Ramos, and R. F. Schwan.(2012). Microbiological and Physicochemical Characterization of Small-Scale Cocoa Fermentations and Screening of Yeast and Bacterial Strains, To Develop a Defined Starter Culture. Applied and Environmental Microbiology 78: 5395–5405.*
4. *Dubernet, S., N. Desmasures and M. Gueguen. (2002). A PCR based method for identification of lactobacilli at the genus level. FEMS Microbiology Letters,* ***214****: 271-275.*
5. *Guimaraes, T. M., D. G. Moriel, I. P. Machado, C. M. T. Fadel Picheth and T. M. B. Bonfim. (2006). Isolation and characterization of Saccharomyces cerevisiae strains of winery interest. Brazilian Journal of Pharmaceutical Sciences,* ***42****: 120-126.*
6. *Igor M. V. M., C. P. M. Maria, F. D. Whasley, R. D. Disney and F. S. Rosane. (2013). Microbial succession and the dynamics of metabolites and sugars during the fermentation of three different cocoa (Theobroma cacao L.) hybrids. Food Research International,* ***54****: 9–17.*
7. *Josepa, S., M. J. M. Guillamon and J. Cano. (2000). PCR differentiation of Saccharomyces cerevisiae from Saccharomyces bayanus/Saccharomyces pastorianus using speci¢c primers. FEMS Microbiology, 193: 255-259.*
8. *Kantor, A., J. Petrova and M. Kacaniova. (2014). Acetobacter aceti Fast Identification by Real Time PCR in Spoiled Wine Samples. Animal Science and Biotechnologies, 47 (2): 94-100.*

9. *Lefeber, T., Z. Papalexandratou, W. Gobert, N. Camu, L. De Vuyst. (2012). On-farm implementation of a starter culture for improved cocoa bean fermentation and its influence on the flavour of chocolates produced thereof. Journal of Food Microbiology,* ***30****:379-392.*

10. *Meersman, E., J. Steensels, M. Mathawan, P. J. Wittocx, V, Saels, N. Struyf, H. Bernaert, G. Vrancken and K. J. Verstrepen.(2013). Detailed Analysis of the Microbial Population in Malaysian Spontaneous Cocoa Pulp Fermentations Reveals a Core and Variable Microbiota. Journal of PLoS ONE,* ***8(12):*** *e81559.*

11. *Ouattara. H. G., B. L. Koffi., G. T., Karou, A. Sangare, S. L. Niamke and J. K. Diopoh. (2008). Implication of Bacillus sp. in the production of pectinolytic enzymes during cocoa fermentation. World J. Microbiol. Biotech.* ***24****:1753-1760.*

12. *Poblet, M., N. Rozes, J. M. Guillamon and A. Mas. (2000). Identification of acetic acid bacteria by restriction fragment length polymorphism analysia of a PCR-amplified fragment of the gene coding for 16S rRNA. Journal of Applied Microbiology,* ***31****: 63-67.*

13. *Schwan, R. F and A. E. Wheals. (2004). The microbiology of cocoa fermentation and its role in chocolate quality. Critical Reviews in Food Science and Nutrition,* ***44****: 205–221.*

14. *Schwan, R. F. (1998). Cocoa Fermentations Conducted with a Defined Microbial Cocktail Inoculum. Applied and Environmental Microbiology .****64(4):*** *1477–1483.*

15. *Schwan, R. F., A. H. Rose, R. G. Board. (1995). Microbial fermentation of cocoa beans, with emphasis on enzymatic degradation of the pulp. Journal of Applied Bacteriology Symposium Supplement,* ***79****:96–107.*

16. *Ukwo, S. P and C. F. Ezeama. (2011). Studies on Proliferation of Acetic Acid Bacteria during Soursop Juice Fermentaion. Internet Journal of Food Safety,* ***13****: 345-350.*

17. *Walter. J., G. W. Tannock, A. Timisjarvi, S. Rodtong, D. M. Loach, K. Munro and T. Alatossava. (2000). Detection and identification of gastrointestinal Lactobacillus species by using denaturing gradient gel electrophoresis and species- specific PCR primers. Applied Environmental Microbiology,* ***66****: 297–303.*